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TITLE: In Vivo Estradiol, Tamoxifen and Raloxifene Modulation of Association/Dissociation Kinetics for Estrogen Receptor, Interacting Co-Factors and DNA Binding Sites

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**13. ABSTRACT** *(Maximum 200 Words)*

This one-year, concept award was approved for the development of quantitative fluorescence imaging technologies that measure ligand-regulated co-localization and interactions of estrogen receptor with its interacting factors. DAMD support enabled us to successfully quantify the extent of factor co-localization using computer-assisted techniques. We also applied these quantitative techniques to accurately measure the extent of energy transfer, between fluorophores as a marker of the extent of interaction between attached molecules. One manuscript was published (see Appendix I) that detailed the effects of estradiol, tamoxifen, and four other ligands on the direct interactions of estrogen receptor with specific protein targets in living cells. A second manuscript is currently being written that expands this analysis to more interacting targets and more ligands. To date, we have used these technologies to identify at least two novel estrogen receptor ligands that regulate estrogen receptor interactions distinct from those regulated by current Selective Estrogen Receptor Modulators, including tamoxifen and raloxifene. Thus, DAMD support permitted the successful development of technologies that dramatically improve the identification and characterization of next-generation, anti-breast cancer reagents.

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## INTRODUCTION

A subset of breast tumors depends upon circulating estrogens for their growth (1, 2). Drugs, such as tamoxifen, which bind the estrogen receptor (ER) to occlude estrogen binding, have been successful in the treatment of this class of breast tumor (3-5). However, these drugs have significant side effects owing to their anti-estrogenic effects in other tissues (5-12). In addition, some of these drugs activate, instead of block, ER action in other tissues in which anti-estrogenic behavior is preferred clinically (3, 5, 7, 13). It is our goal to understand the molecular and cellular basis of the tissue-specific actions of these selective estrogen receptor modulators (SERMs). To that end, it was our purpose to develop quantifiable techniques that permit the imaging, in different cell types, the effects of estrogens and existing SERMs on the molecular actions of ER.

## BODY

This Concept Award was submitted for fiscal year 1999 and approved for funding in fiscal year 2000. There was no formal "Statement of Work" associated with this one-year proposal. However, as per my abstract submitted for final approval on January 9, 2001 "Our immediate goal is to develop cellular assays that discern in unprecedented detail the variations in molecular events that occur upon estradiol, tamoxifen and raloxifene binding to ER...We now aim to develop technologies to quantify and automate these measurements. This is a first step towards adapting this technology for the rapid screening of large drug libraries for compounds of highly selective estrogenic and anti-estrogenic effects."

With DAMD support, we successfully completed the above goals, and more. Analytical techniques were developed that permitted the accurate quantification, in living cells, of the amounts of fluorescence emitted from multiple, spectrally-distinct, fluorophores attached to ER and ER-interacting proteins (14). These quantitative techniques developed to successfully measure co-localization also proved essential for further directions that we pursued (15) (five manuscripts either under review or soon to be submitted). With accurate quantification of fluorescence, we also were able to measure extent of interaction between the fluorophore-tagged molecules as the extent to which energy was transferred from one fluorophore to another, instead of being emitted as light (14). This procedure was developed under this funding and was added to all of the data collection and analytical protocols developed in these experiments.

We initially proposed to conduct these experiments using ER tagged with the blue fluorescent protein (BFP) and ER-interacting targets fused with the green fluorescent protein (GFP). These BFP and GFP fusions had been used by us in initial experiments examining the principle of the co-localization technique (16). The relatively instability of BFP, together with the advent of a red fluorescent protein (RFP) allowed us to modify the procedure early in the project. Thus, the protocol was modified slightly to include the construction ER-RFP fusion proteins, which were then used in conjunction with GFP-linked ER-interacting co-factors for the analyses that were successfully completed (14).

The success of the procedures relied on computer-assisted data analysis. We constructed a string of computer commands that allowed us to:

1) quantify the extent of co-localization between different fluorophores as correlation co-efficients based upon background-subtracted fluorescence measurements from individual pixels;
2) quantify the amount of fluorescence resonance energy transfer (FRET) from a donor fluorophore to an acceptor fluorophore, again at individual pixels within each image;
3) quantify the amount of energy transfer, normalized to the amounts of acceptor and donor present at each pixel; this normalized FRET provides information about
   a) the interaction kinetics of ER and its interacting partners
   b) the distance separating the fluorophores
   c) the rotational constraints imparted to the fluorophore by the attached proteins;
4) quantify the amounts of fluorescence at marked structures within the cell and compare the co-localization and direct interactions (measured by FRET) at different structures.

In addition to developing the analytical techniques required for the success of these powerful techniques, DAMD support has allowed us to automate these procedures. We are currently performing a complete analysis of all elements discussed above from a single cell in less than 10 seconds. All data are automatically downloaded into a spreadsheet, from which the data from hundreds of cells, from multiple independent experiments are statistically compared. We are currently engaged in a non-proprietary collaboration with Universal Imaging Corporation (Downingtown, PA) to make these techniques more user-friendly and widely available to the scientific community.

Initial work outlining the FRET technique, applied to total fluorescence emitted from cells, has recently been published (14) (see Appendix 9). Further, pixel-by-pixel analyses of co-localization and FRET regulated by ligands are soon to be submitted in two separate manuscripts, one by Dr. Xin Lu, whose salary was supported with DAMD17-1-01-0498 funding, and the other by Raphael Calmon, who assisted in the generation of the automated commands.

## KEY RESEARCH ACCOMPLISHMENTS

All of the accomplishments listed below are complete and have either been accepted for publication, or are currently being written for publication:

1) Estradiol, tamoxifen, raloxifene, faslodex (ICI 182,780), diethylstilbestrol differentially regulate the interactions of ER with different ER-interacting co-factors in living cells (14).
2) One novel ER ligand, 6, 4'-dihydroxyflavone, synthesized in the laboratory of the collaborating laboratory of Thomas S. Scanlan, showed a highly specific enhancement of the interaction of ER with one specific target but not another related target molecule (14). This demonstrated the extent to which our techniques are able to tease out subtle differences in the interactions regulated by specific SERMs in living cells.
3) The novel SERMs found to regulate interactions within living cells were also found to activate ER-dependent transcription in more traditional experimental analyses (14).

4) Estradiol, tamoxifen, 4-hydroxy-tamoxifen and raloxifene all promote dimerization of estrogen receptor alpha in living cells.
5) ER-alpha dimerization is promoted better by faslodex than by the other compounds listed in bullet 4.
6) ER-alpha dimerization is promoted more poorly by the soy isoflavone genistein than by the other compounds listed in bullet 4.
7) All of the compounds listed in bullets 4-6 promote the same amount of hetero-dimerization between ER-alpha and ER-beta, despite their different effects on ER-alpha homo-dimerization.
8) Four novel, chemical derivatives of tamoxifen behave exactly as tamoxifen and 4-hydroxy tamoxifen in promoting ER-alpha homo-dimerization.
9) One chemical derivative of genistein behaves exactly as genistein in being a poor activator of ER-alpha homo-dimerization.
10) Another modification of genistein (containing one additional attached phenyl group) is converted to promote ER-alpha dimerization as effectively as estradiol, tamoxifen, 4-hydroxy-tamoxifen, the tamoxifen derivatives or raloxifene. This acts as a chemical marker for the types of functional groups and interactions that affect how a compound regulates isoform-selective dimerization in living cells.

## REPORTABLE OUTCOMES

One manuscript, derived from the initial parts of our work (bullet points 1-3 above) and that acknowledges DAMD17-01-1-0498 support, has already been published (14) (see Appendix 9).

The data for at least two additional manuscripts have already been collected and statistically analyzed to completion. These data are currently in preparation (bullet points 4-10) and will acknowledge DAMD17-01-1-0498 support.

Research supported from DAMD17-01-1-0498 support, has also been reported in a number of talks by the Principal Investigator (Appendices 1-8) including:
-An invited lecture at the University of Michigan, Ann Arbor, MI, December 12, 2001;
-An invited lecture at the California Breast Cancer Research Program annual meeting, Oakland, CA, March 9, 2002;
-An invited lecture at the American Association for Cancer Research annual meeting, San Francisco, CA, April 8, 2002;
-An invited lecture at the Keystone Symposium on Nuclear Receptor Function, Snowbird, UT, April 14, 2002;
The Principal Investigator will also include this data in invited talks scheduled for June at the Susan G. Komen Breast Cancer Foundation Meeting (Washington, DC) and at the annual meeting of the Endocrine Society (San Francisco, CA)

The FRET techniques developed with this small Concept proposal are now forming the basis of a number of different projects in a number of grant applications. This includes:
-a new DAMD application that follows up this ER research work,

-two new applications to the National Institutes of Health on parallel studies of other nuclear receptors (the class of protein to which ER belongs),
-a renewal application to the National Institutes of Health for work on the molecular interactions involved in pituitary cell differentiation,
-a shared instrumentation grant application to the National Institutes of Health for the purchase of equipment that will allow co-localization and FRET to be measured in three dimensions and with time, and
-a UCSF intramural application studying the molecular interactions of factors involved in adipogenesis.
-The FRET techniques developed with this small Concept proposal also have been incorporated into a number of other ongoing, breast cancer-related studies involving the estrogen receptor that are currently funded (DAMD17-1-01-190 and from the Susan G. Komen Foundation).

## PERSONNEL RECEIVING PAY FROM DAMD17-1-01-0498

| | |
|---|---|
| Xin Lu, M.D., Ph.D.: | Post-Doctoral Scientist |
| Catherine Price, B. S.: | Staff Research Associate II |

## REPORTS AND PUBLICATIONS FROM DAMD17-1-01-0498 FUNDING

All current reports and publications are included in Abstracts 1 to 10. Please keep in mind that future publications are currently being written from the results of experiments supported by DAMD17-1-01-0498 funding.

## CONCLUSIONS

With the completion of this project, we can now directly measure biochemical interactions in living cells. The significance of this is that we may now compare similar interactions under different cellular conditions to gain an understanding of the mechanisms by which SERMs regulated ER action cell-specifically. Furthermore, the technique itself is amenable to virtually any study of interactions between molecules. As such, it is sure to find wide general use for the elucidation of virtually any biomolecular pathway involved in any disease state.

Although we have successfully developed the technique, there are still directions that we intend to embark upon in the future. One direction is to follow the interactions with time after ligand addition. Initial experiments were attempted but failed due to the difficulties of keeping a three-dimensional cell in two-dimensional focus during the automated collection period. This is being overcome by imaging automatically in multiple focal planes and reconstructing the images in three-dimensions over time (four-dimensional imaging). Finally, techniques are now becoming available in which cells marked with fluorophores can be studied *in situ* in living mice. We have already contacted laboratories with such equipment and look forward to applying our FRET techniques on whole animals to study organ-selective interactions between ER and its interacting factors.

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## APPENDICES

1-8 . Abstracts for meetings in which supported data was, or is to be, discussed.

9. Published Manuscript acknowledging support from DAMD17-1-01-0498:

Weatherman RV, Chang C-Y, Clegg NC, Carroll DC, Day RN, Baxter JD, McDonnell DP, Scanlan T, Schaufele F 2002 Ligand-dependent interactions of estrogen receptor detected in vivo by Fluorescence Resonance Energy Transfer. Mol Endocrinol 16: 487-496

# APPENDIX 1

Abstract for California Breast Cancer Research Program Meeting. Poster and talk presented March 9, 2002 in Oakland, CA

**Novel Technologies to Identify Tissue-Selective Estrogens**

Fred Schaufele (poster presenter), Xin Lu, Catherine Price, University of California, San Francisco

Breast tumors are broadly classified into two categories: those that contain, or do not contain, proteins that bind estrogens. Breast tumors that contain these proteins, called "estrogen receptors", grow in the presence of an estrogen. The estrogen receptor does not function by itself but rather works by interacting with other proteins called "co-factors". The types of co-factors that interact with the estrogen receptor change when estrogen binds the estrogen receptor. Thus, the estrogen-regulated interaction of co-factors with the estrogen receptor determines whether the tumor cell grows or not.

Certain drugs, including tamoxifen, block the growth of estrogen receptor-containing tumors. These drugs bind to the estrogen receptor and cause it to behave like the estrogen receptor without estrogen. However, some co-factors that interact with the estrogen receptor bound by tamoxifen also interact with the estrogen receptor bound by estrogen. Thus, tamoxifen blocks some co-factor interactions, particularly those required for the growth of breast tumors, but allows other co-factor interactions that cause the growth of some other tissues. In patients treated with tamoxifen, there is an increased risk of tumors in those other tissues, particularly the uterus.

Defining these co-factor interactions is the key to developing improved drugs not containing side effects. We developed novel cellular imaging technologies to follow the estrogen- and tamoxifen-induced changes in estrogen receptor interactions with itself and with specific co-factors in living cells. Briefly, we introduce into cells estrogen receptor and interacting proteins marked with different dyes that are activated by light of specific wavelengths to emit light of longer wavelengths. The activation and emission wavelengths of the different dyes partially overlap such that they cross-talk with each other. The degree of cross-talk tells us whether the tagged molecules interact and, if they do, how they interact and what shape the molecule attains.

Our long-term goal is to associate specific estrogen receptor/co-factor interactions with the desirable and undesirable clinical effects of each drug, then use this detailed knowledge to identify novel drugs that affect different subsets of those interactions. We present here our work, to date, characterizing these estrogen receptors interactions promoted or blocked by a number of clinically proven drugs and novel compounds. Ultimately, compounds with highly selective co-factor specificities may be identified that improve breast cancer treatment and/or minimize the side effects of current therapies. An ideal SERM could even be administered for decades to prevent breast cancer.

# APPENDIX 2

Abstract for American Association for Cancer Research Annual Meeting. Talk presented April 8, 2002 in San Francisco, CA

**Cellular imaging to identify ligands that modulate selected estrogen receptor actions**

Fred Schaufele, Catherine Price, Xin Lu, University of California, San Francisco, San Francisco, CA.

Breast tumors are broadly classified into two categories: those that contain, or do not contain estrogen receptors (ER). In ER-containing tumors, growth occurs when estrogens bind to, and change the conformation of, ER. Tamoxifen, which is effective in slowing the growth of estrogen-dependent tumors, also binds ER, but causes it to change to a different conformation. These different ER conformations result in different co-factor interactions with the tamoxifen- or estrogen-bound ER such that tamoxifen acts as an anti-estrogen in some tissues but as an estrogen in others. We developed novel imaging technologies to follow the estrogen- and tamoxifen-induced changes in ER interactions with specific co-factors in living cells. We labeled the two known ERs and each co-factor with fluorescent tags of different colors and transferred them into cells. Fluorescence from each receptor and co-factor was quantified within 100 x 100 nm sections of the cell. Following treatment with estradiol, tamoxifen or 15 other "selective estrogen receptor modulators" (SERMs), we determined if the co-factor became more or less concentrated at the location of the estrogen receptor and if the proteins were so close that energy transferred between the attached fluorophores. We identified a number of different effects of different ER ligands on ER conformation and interaction. Each ligand modulated selected ER activities, but our measurement of these activities within living cells further distinguished spatial and temporal components of each ligand's action. Some ligands modulated ER conformation or co-factor interactions within localized regions of the nucleus. Other ligands modulated the number of sites within the nucleus at which specific conformations or interactions were detected. Our long-term goal is to associate specific ER conformation and co-factor interactions with the desirable and undesirable clinical effects of each drug. This detailed knowledge will be used to identify novel drugs that affect different subsets of those interactions. Some of these highly selective compounds may improve breast cancer treatment and/or minimize the side effects of current SERM therapies. An ideal SERM could even be administered for decades to successfully prevent breast cancer and to provide risk-free estrogen replacement therapy for post-menopausal women.

# APPENDIX 3

Abstract for Keystone Symposium, Nuclear Receptor Meeting. Talk presented April 14, 2002 in Snowbird, UT.

**Cellular imaging of nuclear receptor action**

F. Schaufele, X. Lu, C. Price, R. Calmon
University of California, San Francisco, San Francisco, CA

We developed novel imaging technologies to follow ligand-regulated changes in nuclear receptor (NR) conformation, dimerization or interactions with specific co-factors in living cells. Estrogen receptors (ER) and multiple NR-interacting co-factors were labeled with different fluorescent tags and expressed in cells. Fluorescence from each receptor and co-factor was quantified within 100 x 100 nm sections of the cell in the presence or absence of different ligands to determine which ligand caused which co-factor to concentrate at the location of the ER. Direct interaction of ER and the interacting co-factor was measured by fluorescence resonance energy transfer (FRET). The effect of ligand on ER conformation and dimerization similarly was measured by studying FRET between the ERs themselves. Some ligands altered the number of sites within the nucleus at which FRET was observed. Other ligands changed the efficiency of FRET detected at each site, indicating that the conformation of the dimer or of ER/co-factor interaction was changed. In contrast, TR homodimers were limited to a small number of subnuclear regions and were disrupted by thyroid hormone. Instead, TR preferred to formed heterodimers with RXR in a ligand-independent fashion. Thus, the spatial and temporal characteristics of ligand regulation of NR conformation, dimerization and co-factor interaction can be determined in the physiologic environment of the living cell. This is correlated with the known transcriptional and clinical effects of each NR ligand to identify, with unprecedented detail, the molecular and cellular events underlying nuclear receptor action.

# APPENDIX 4

Abstract for the U.C.S.F. Center for Reproductive Sciences Annual Retreat. Talk to be presented April 29, 2002 in Tiburon, CA.

**Estrogen Receptor Dimerization and Interactions in Living Cells**
Fred Schaufele, Xin Lu, Catherine Price

Estrogens regulate a number of tissues including those involved in reproductive functions. Women who have undergone menopause, or other estrogen-deficient patients, experience a decline in overall health related to the absence of tissue-selective regulation of the nuclear estrogen receptor (ER). Hormone replacement therapies are possible. However, complications arising from estrogen replacement include an increased risk of breast and endometrial cancers as well as venous thrombosis. As a result, long-term estrogen replacement therapies have not become widespread despite their potential for significantly improving overall health and quality of life.

An ideal long-term estrogen replacement therapy would block ER actions in specific tissues while promoting ER action in other tissues. This requires methods for detecting the fine details of ER action in different cell types in response to each selective ER modulator (SERM). We developed novel fluorescence microscopy techniques for studying ER interaction within living cells. ER and ER-interacting factors are tagged with red and green fluorophores and their relative intracellular positions are determined upon incubation with SERMs. The extent to which energy is transferred from the green fluorophore to the red fluorophore identifies direct interactions between ER and each co-factor at specific intracellular locations within each cell type examined.

Using our fluorescence co-localization and resonance energy transfer (FRET) techniques, we determine, for example, that some ligands, including estradiol, tamoxifen and the soy isoflavone genistein, all increase the number of intranuclear sites at which ERα homodimers and ERα/ERβ heterodimers are found. FRET measurements also show that the estradiol and tamoxifen-bound dimers are kinetically, or conformationally, different from the genistein-bound dimers. In contrast, estradiol, tamoxifen and genistein each promote interactions and co-localization with different subsets of co-factors. Our goal is to establish a "fingerprint" of the conformational and interactive consequences of each ligand to the ER in different ER-responsive tissues. These fingerprints will be compared to the known clinical effects of each compound. This will aid the identification of ER ligands with unique tissue-selective activities, some of which may prove useful for safe, long-term, post-menopausal hormone replace therapies. Some of these ligands may even find a role in pre-menopausal breast and uterine cancer prevention.

# APPENDIX 5

Abstract for the Susan G. Komen Breast Cancer Foundation Meeting. Talk to be presented June 3, 2002 in Washington, DC.

**Cellular Imaging to Identify SERMs Improved for Breast Cancer Therapy**

Fred Schaufele, Xin Lu, Catherine Price

Breast tumors are broadly classified into two categories: those that contain, or do not contain, proteins that bind estrogens. Breast tumors that contain these proteins, called "estrogen receptors", grow in the presence of an estrogen. The estrogen receptor does not function by itself but rather works by interacting with other proteins called "co-factors". The types of co-factors that interact with the estrogen receptor change when estrogen binds the estrogen receptor. Thus, the estrogen-regulated interaction of co-factors with the estrogen receptor determines whether the tumor cell grows or not.

Certain drugs, including tamoxifen, block the growth of estrogen receptor-containing tumors. These drugs bind to the estrogen receptor and cause it to behave like the estrogen receptor without estrogen. However, some co-factors that interact with the estrogen receptor bound by tamoxifen also interact with the estrogen receptor bound by estrogen. Thus, tamoxifen blocks some co-factor interactions, particularly those required for the growth of breast tumors, but allows other co-factor interactions that cause the growth of some other tissues. As a result, in patients treated with tamoxifen, there is an increased risk of tumors in those other tissues, particularly the uterus.

Defining these co-factor interactions is the key to developing improved drugs not containing side effects. We developed novel cellular imaging technologies to follow the estrogen- and tamoxifen-induced changes in estrogen receptor interactions with specific co-factors in living cells. Our long-term goal is to associate specific estrogen receptor/co-factor interactions with the desirable and undesirable clinical effects of each drug, then use this detailed knowledge to identify novel drugs that affect different subsets of those interactions. Ultimately, compounds with highly selective co-factor specificities may be identified that improve breast cancer treatment and/or minimize the side effects of current therapies. An ideal SERM could even be administered for decades to prevent breast cancer.

# APPENDIX 6

Abstract for the Endocrine Society Annual Meeting. Talk to be presented June 22, 2002 in San Francisco, CA.

**Conformation and Interactions of Transcription Co-Regulatory Factors at Discrete Subnuclear Loci Revealed by FRET Nanoscopy**

Fred Schaufele, Xin Lu, Raphael Calmon
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Austin College, Sherman, TX, 75090
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University of Virginia, Charlottesville, VA, 22908-0578

Transcription factors direct co-regulatory complexes to genes buried within the genome. Compartmentalization of gene regulatory factors in the highly organized nucleus may affect their structure at and/or access to specific genes. By fluorescence microscopy, we determined the subnuclear locations of a number of transcription factors and co-regulatory factors that control gene expression and differentiation in multiple cell types. Expression of the transcription factor C/EBP[alpha], which is absent from pituitary and adipocyte progenitor cells, was accompanied by a highly selective recruitment of the co-activator CBP and acetylated histone H3 to the peri-centromeric chromatin, where C/EBP[alpha] concentrated. In the pituitary model, the expression of a second transcription factor, Pit-1, dispersed C/EBP[alpha] away from the peri-centromeric chromatin. Nuclear receptors also altered the location of specific co-regulatory factors, in a ligand-regulated fashion.

To study the corresponding biochemical events associated with intranuclear reorganization, we developed fluorescence resonance energy transfer (FRET) techniques that precisely measure the conformations and interactions of co-localized molecules at each of thousands of sites within living cells. C/EBP[alpha] formed dimers effectively at most locations throughout the nucleus. Tagging different domains of C/EBP[alpha], and measuring the amount of inter-domain FRET, demonstrated that the conformation of C/EBP[alpha] was different at the peri-centromeric chromatin than in the rest of the nucleus. Treating the cells with an activator of protein kinase C changed the structure of C/EBP[alpha]. For some nuclear receptors, we found that dimers were distributed unevenly throughout the nucleus. Cognate ligands increased (estrogen receptor [alpha] and [beta] homo and heterodimers), decreased (thyroid hormone receptor homodimer) or had little effect on (RXR[alpha] homodimer, TR[beta]/RXR[alpha] heterodimer) the number of subnuclear sites at which interactions occurred. Some ligands also changed the extent of interaction at each site. For the ER, we found differences in the types of dimerizations and interactions promoted by different ligands used in breast cancer therapy. Thus, the interactions of conformations of gene regulatory complexes are determined by intranuclear location. The re-location of co-regulatory factors by some transcription factors may regulate the patterns of gene expression in differentiation.

# APPENDIX 7

Abstract submitted for the CDMRP Breast Cancer Research Program Meeting, September 25-28, Orlando, FL.

**Cellular Imaging Technologies for Identifying Tissue-Selective SERMS**

Fred Schaufele, Catherine Price, Xin Lu

Tamoxifen is an estrogen receptor (ER)-binding compound effective for blocking the proliferation of many ER-containing breast tumors. With its anti-estrogenic effects, tamoxifen blocks also the beneficial effects of estrogens in some tissues. Tamoxifen also acts like an estrogen in some tissues in which anti-estrogenic activities would be preferred. These undesired estrogenic and anti-estrogenic side effects limit the use of tamoxifen for breast cancer prevention only to high-risk patients.

An ideal treatment for breast cancer would block ER actions in specific tissues while promoting ER action in other tissues. This requires methods for detecting the fine details of ER action in different cell types in response to each selective ER modulator (SERM). We developed novel fluorescence microscopy techniques for studying ER interaction within living cells. ER and ER-interacting factors are tagged with red and green fluorophores and their relative intracellular positions are determined upon incubation with SERMs. The extent to which energy is transferred from the green fluorophore to the red fluorophore identifies direct interactions between ER and each co-factor at specific intracellular locations within each cell type examined.

Using our fluorescence co-localization and resonance energy transfer (FRET) techniques, we determine, for example, that some ligands, including estradiol, tamoxifen and the soy isoflavone genistein, all increase the number of intranuclear sites at which ERα homodimers and ERα/ERβ heterodimers are found. FRET measurements also show that the estradiol and tamoxifen-bound dimers are kinetically, or conformationally, different from the genestein-bound dimers. In contrast, estradiol, tamoxifen and genestiein each promote interactions and co-localization with different subsets of co-factors. Our goal is to establish a "fingerprint" of the conformational and interactive consequences of each ligand to the ER in different ER-responsive tissues. These fingerprints will be compared to the known clinical effects of each compound. This will aid the identification of ER ligands with unique tissue-selective activities, some of which may prove useful for safe, long-term breast cancer prevention therapies.

# APPENDIX 8

Abstract submitted for the CDMRP Breast Cancer Research Program Meeting, September 25-28, Orlando, FL.

**Kinetics of Estrogen Receptor Action in Cell Types Relevant to Breast Cancer**
Fred Schaufele, Xin Lu

The proliferation of a subset of breast tumors depends upon circulating estrogesn. These tumors generally contain the estrogen receptor (ER), which is a target for the anti-breast cancer drug tamoxifen. However, tamoxifen is not an ideal drug, and its side effects in non-breast tissues limit its use in preventative therapies.

Our goal is to understand the tissue-selective actions of estrogen action. We have developed novel fluorescence imaging techniques that precisely define the molecular actions of ERa and ERb in cultured tumor cells. These techniques apply a physical principal, in which energy is transferred from a fluorophore ("GFP") attached to ER to a second fluorophore ("RFP") attached to nearby ER. This has allowed us to precisely track ER dimerization in living cells. With these techniques, we have characterized the effects on ER dimerization of estradiol and certain selective estrogen receptor modulators (SERMs), including tamoxifen, raloxifene, ICI 182780, the soy isoflavone genestein, multiple derivatives of genestein and diethylstilbestrol.

At individual pixels within each image captured from a cell, we quantify the amount of ER-GFP, ER-RFP and energy transfer (FRET). We also calculate the amount of FRET normalized for the amounts of ER-GFP and ER-RFP present. This "efficiency" of FRET reflects the amount of contact between ER within the dimer and informs us of the kinetics of the interaction.

In the absence of ligand, there is some FRET indicating some dimerization. In the presence of any of the above ligands, the amount of FRET increases. In particular, for genestein and derivatives, the amount of FRET increases because there is an increase in the proportion of pixels (i.e. sites within the nucleus) at which ER dimerizes. This increase in the number of dimerization sites also was observed for the other ligands. For these ligands, there also was an increase in FRET efficiency at each site. Thus, these ligands also alter the dimer interaction itself. We also have applied FRET to identify SERM-selectivity in ER interactions with target proteins. By mapping fine distinctions between the molecular actions of each SERM, we aim to identify novel SERMs with improved specificity for breast cancer treatment and prevention.

# APPENDIX 9

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# Ligand-Selective Interactions of ER Detected in Living Cells by Fluorescence Resonance Energy Transfer

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**Some aspects of ligand-regulated transcription activation by the estrogen receptor (ER) are associated with the estrogen-dependent formation of a hydrophobic cleft on the receptor surface. At least *in vitro*, this cleft is required for direct interaction of ER with an $\alpha$ helix, containing variants of the sequence LXXLL, found in many coactivators. In cells, it is unknown whether ER interactions with the different LXXLL-containing helices are uniformly similar or whether they vary with LXXLL sequence or activating ligand. Using fluorescence resonance energy transfer (FRET), we confirm in the physiological environment a direct interaction between the estradiol (E2)-bound ER and LXXLL peptides expressed in living cells as fusions with spectral variants of the green fluorescent protein. This interaction was blocked by a single amino acid mutation in the hydrophobic cleft. No FRET was detected when cells were incubated with the antiestrogenic ligands tamoxifen and ICI 182,780. E2, diethylstilbestrol, ethyl indenestrol A, and 6,4′-dihydroxyflavone all promoted FRET and activated ER-dependent transcription. Measurement of the level of FRET of ER with different LXXLL-containing peptides suggested that the orientations or affinities of the LXXLL interactions with the hydrophobic cleft were globally similar but slightly different for some activating ligands. (*Molecular Endocrinology* 16: 487–496, 2002)**

THE ERα AND ERβ ARE members of a large class of nuclear receptors that regulate the transcription of genes in response to binding small molecule ligands (1–3). The regulatory roles of ER in disorders like breast cancer and osteoporosis make it an important therapeutic target (4–9). One of the signature features of the ER-targeting compounds is that they may have different stimulatory or repressive effects depending on the cellular context. For instance, the breast cancer drug tamoxifen is an antiestrogen in breast tissue but, in the uterus, it mimics the estrogenic activity of the physiological hormone, E2 (7, 10). Other compounds, such as the osteoporosis drug raloxifene, show a different clinical profile (11). Improved designer estrogens with higher selectivity for specific tissues would permit tissue-specific, estrogen-regulated disorders to be treated with minimal side effects (12, 13).

The mechanisms by which E2 and the selective ER modulators (SERMs) show tissue-specific activities remain unresolved but are at least partially related to ligand-regulated alterations in ER structure and function. In both its unliganded and liganded state, the ER is part of larger complexes with other accessory proteins (14). These accessory proteins, some of which are still unidentified, can stabilize ER structure and regulate transcription at different DNA effector sites (15). Ligand binding causes a conformation change in the ER (16, 17), which alters the affinities of the receptor for these accessory proteins (18, 19). One possible mechanism for SERM activity is that different ligands recruit different sets of accessory proteins and thereby differentially regulate gene transcription (20–22). Differential cofactor interactions, together with tissue-dependent expression of ERα, ERβ, and each cofactor, could explain tissue-selective SERM activity.

To elucidate, and ultimately predict, differential SERM action, it is therefore essential to measure the ligand-induced, direct interactions between the ER and different accessory proteins in the cellular environment (20–22). Many coactivators that interact with the E2-activated ER contain one or more copies of a consensus sequence, LXXLL (L, leucine; X, any amino acid) (23, 24). Structural studies have shown that an

Abbreviations: CFP, Cyan fluorescent protein; DES, diethylstilbestrol; DHF, 6,4′-dihydroxyflavone; EIA, indenestrol A; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; L, leucine; RFP, red fluorescent protein; SERM, selective ER modulator; X, any amino acid.

isolated LXXLL peptide will interact with a hydrophobic cleft that forms on one surface of the E2-bound ER (16). This hydrophobic cleft constitutes the activation function AF-2, which is conserved amongst nuclear receptors and participates in ligand-regulated gene transcription (25). Two-hybrid interaction assays have proved very useful for identifying and characterizing the ligand-regulated interactions of LXXLL-containing factors and peptides with ER expressed in cells (20–22). However, two-hybrid assays measure only whether proteins interact, and not whether they interact with differing structural characteristics or affinities.

We applied a microscope-based assay using fluorescence energy resonance transfer (FRET) to measure in living cells the ability of a ligand to modulate LXXLL interactions with ER. FRET measures the proximity of two molecules as a consequence of the degree to which the fluorescence energy excited in a donor fluorophore, linked to one factor, is not emitted and instead is nonradiatively transferred to an acceptor fluorophore, linked to another factor (26–30). We observed in the cellular environment that ERα, fused to the red fluorescent protein (RFP) interacted directly with LXXLL peptides, fused to the cyan (CFP) or green (GFP) fluorescent proteins. These interactions were promoted by E2 but blocked by tamoxifen and another SERM, ICI 182,780, which confirmed prior studies in two-hybrid (21) and fluorescence colocalization (31) assays. Like E2, the synthetic ligands diethylstilbestrol (DES), ethyl indenestrol A (EIA), and 6,4′-dihydroxyflavone (DHF) promoted FRET between ERα-RFP and two different LXXLL peptides fused to GFP. All these interactions were dependent upon the integrity of AF-2 within the ligand binding domain of ERα. E2, DES, EIA, and DHF yielded similar levels of FRET for the interaction of ER with one LXXLL peptide. However, small, ligand-selective differences in the level of FRET were measured for interaction with the other LXXLL target sequence. This indicated that there were subtle, ligand-specific, and LXXLL-specific differences in the orientation or affinity of LXXLL interaction with ER. The accurate measurement of such nuances in the interactions of ER in the cellular environment will help distinguish the similarities and cell-type dependent differences in ligand-selective ER activities.

## RESULTS

### Fluorescent Protein-Tagged ERα and LXXLL for FRET Measurements

Isolated LXXLL sequences retain the ability to interact specifically with estrogen-bound ER (16, 21). In our initial studies, the 19-amino acid-long LXXLL-containing sequence F6 (21), previously shown to form a complex with ERα (21, 31), was fused to the carboxy terminus of CFP. X-ray crystallographic structures of LXXLL bound to ER (16) predict that, if LXXLL binds directly to ER, the CFP fluorophore should project toward RFP fused to the carboxy terminus of ERα. This positioning would be optimal for FRET from the CFP donor to the RFP acceptor.

The ERα-RFP fusion was transcriptionally active (Fig. 1). ERα-RFP or control expression vectors were transfected into ER-deficient HepG2 cells together with either of two different, E2-sensitive promoters controlling the expression of a luciferase reporter. One promoter consisted of three copies of an estrogen response element linked to a minimal TATA box (32). This reporter defines the "classical" activities of ERα-RFP, in which estrogen response is mediated by direct ER interaction with a single DNA binding site in the promoter. The second promoter, from the complement 3 gene (C3), contains three suboptimal ER binding elements, which together allow ER to bind and regulate transcription in response to E2 (33).

Two days after transfection, promoter activity was assessed by measuring the amount of luciferase expressed in extracts of cells grown in E2-deficient media or in parallel cells treated with $10^{-6}$ M E2. Both the 3xERE (Fig. 1, *black bars*) and C3 (Fig. 1, *white bars*) promoters were activated upon E2 addition. In contrast, tamoxifen and ICI 182,780 did not activate ERα-RFP at either promoter, even though wild-type ERα weakly activated the C3 promoter in the presence of tamoxifen, but not ICI 182,780 (22, 33). Thus, ERα-RFP was defective in tamoxifen activation. Because the estrogenic activities of E2 were not disrupted by the fusion of RFP to the carboxy terminus of ERα, ERα-RFP remained viable for studying agonist activation via AF-2.

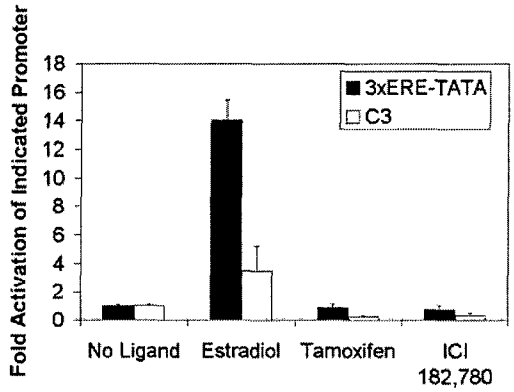



**Fig. 1.** E2-Regulated Activation of Two Promoters by the ERα-RFP Fusion Protein

HepG2 cells, grown in E2-free media, were transfected with the ERα-RFP expression vector and either of two reporter plasmids expressing luciferase under the control of E2-responsive promoters. The 3xERE-TATA and complement C3 promoters were activated upon incubation of the cells with E2, but not by the SERMs tamoxifen or ICI 182,780.

### Controls for the Accurate Measurement of FRET between CFP-LXXLL and ERα-RFP

The measurement of FRET relies on the accurate quantification of the amount of fluorescence emitted by the donor and acceptor fluorophores upon donor excitation (29). The donor CFP is excited optimally by blue light to emit light of energy in the blue-green (cyan) wavelength, whereas the RFP acceptor emits red light upon excitation by light of mid-visible wavelengths, including cyan. If the cyan fluorescent CFP is in close proximity to RFP (<100 Å apart), some of the fluorescence energy from CFP will be absorbed by, and excite, RFP. Thus, when excited by blue light, energy transfer from CFP to RFP would decrease the emission of cyan light and increase the emission of red light.

For controls, we first quantified the amount of fluorescence in cells that independently expressed ERα-RFP or CFP-LXXLL. Expression vectors encoding ERα-RFP or CFP-LXXLL were transfected into GHFT1–5 pituitary progenitor cells grown in estrogen-free media and plated onto microscope coverslips. GHFT1–5 cells contain endogenous ERα, but promoter responses to E2 in GHFT1–5 cells are not significantly altered upon ERα overexpression (34, 35). Because overexpression of ERα in GHFT1–5 cells does not reduce ER response as it does in many other cell types (36), the actions of expressed ER measured in GHFT1–5 cells likely mimic those of endogenous receptors. GHFT1–5 cells also have a flat morphology, which facilitates data collection by fluorescence microscopy (31, 37). The transfected cells were treated with E2, or other ligands as discussed later, or with the control vehicle (ethanol). After allowing 24 h for expression, the amounts of fluorescence emitted from the control cells separately expressing ERα-RFP and CFP-LXXLL were measured in the cyan, red, and FRET channels by quantitative fluorescence microscopy. Digital images from cells expressing CFP-LXXLL were collected by specifically exciting CFP with light of wavelengths between 431 and 434 nm and collecting emissions between 455 and 480 nm (Fig. 2A, *cyan*). Digital images from the ERα-RFP control cells were collected by 550–560 nm excitation and 580–630 nm emission (Fig. 2B, *red*).

The excitation/emission parameters for CFP and RFP resulted in little bleedthrough fluorescence, respectively, in the control cells expressing ERα-RFP and CFP-LXXLL (Fig. 2B, *cyan*; Fig. 2A, *red*). This bleedthrough was accurately quantified by marking each nucleus containing CFP-LXXLL or ERα-RFP as a contiguous assembly of pixels containing more fluorescence than the background. The total amount of cyan and red fluorescence above the background fluorescence was then measured within the nucleus of each control cell. Red fluorescence from 27 different E2-treated cells expressing only CFP-LXXLL was negligible: on average, 0.0009 ± 0.0026 the amount of cyan fluorescence. This means that the amount of

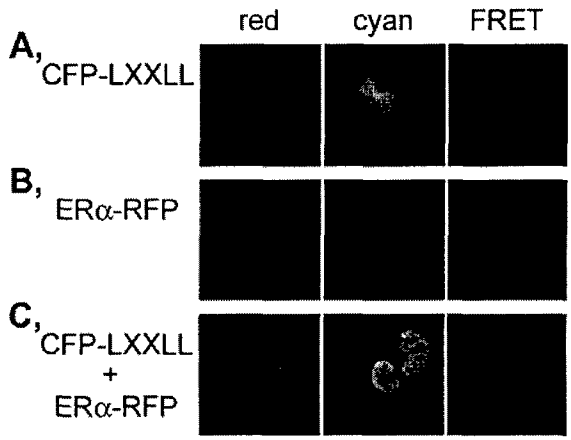



**Fig. 2.** FRET Microscopy of LXXLL Interactions with ERα

GHFT1–5 cells grown in estrogen-free media were transfected with the A, CFP-LXXLL expression vector; B, ERα-RFP expression vector or C, both vectors together, then incubated with E2 (shown) or other ligands (not shown). Digital fluorescent images were collected using *red*-, *cyan*-, or FRET-selective excitation and emission filters. Coexpression of CFP-LXXLL with ERα-RFP causes the LXXLL peptide to occupy the intranuclear location of ERα in estrogen-treated cells (31).

energy emitted by CFP in the red channel was statistically insignificant. Similarly, the bleedthrough of ERα-RFP into the cyan channel was 0.0016 ± 0.0019 the amount of emission in the red' channel. These ratios were similar regardless of the ligand treatment for each cell (not shown). Although the amount of CFP-LXXLL or ERα-RFP expressed in each transiently transfected cell varied, plotting the amount of bleedthrough as a function of the amount of CFP-LXXLL or ERα-RFP fluorescence in each cell (Fig. 3, A and B, *open boxes*) showed that these ratios were consistently measured regardless of the amount of CFP-LXXLL or ERα-RFP expressed.

### Ligand-Regulated FRET between CFP-LXXLL and ERα-RFP

As described previously (31), expression of ERα caused the coexpressed LXXLL peptide to colocalize with the E2-bound ERα, whereas the LXXLL peptide, by itself, distributed throughout the cell (Fig. 2). To determine if there was a hormone-regulated, direct interaction of LXXLL with colocalized ERα, we measured FRET between coexpressed CFP-LXXLL and ERα-RFP. The low level of CFP and RFP bleedthrough enabled us to selectively and accurately measure the amounts of CFP-LXXLL and ERα-RFP coexpressed in the same cell. These values are then used to correct for the contributions of the known amounts of CFP and RFP to the FRET channel, as discussed below.

In cells coexpressing CFP-LXXLL and ERα-RFP, FRET was detected, upon blue light excitation, as an increase in acceptor fluorescence transferred from the

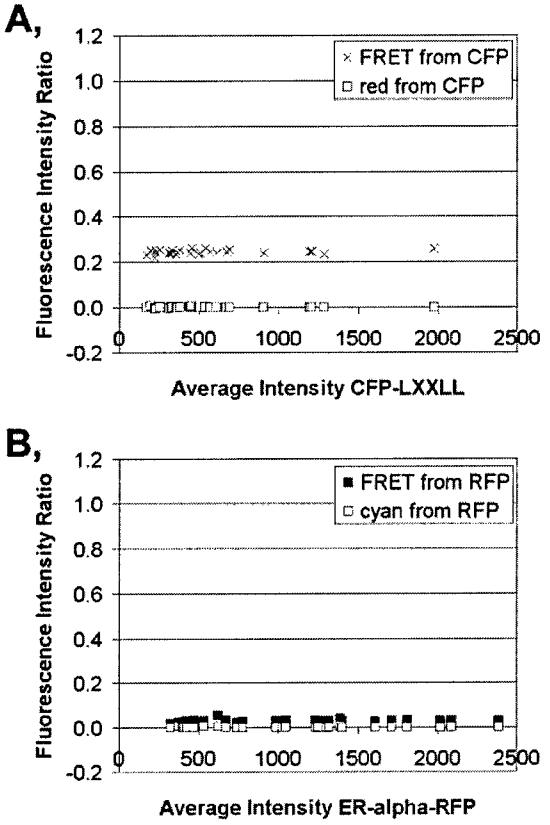



**Fig. 3.** Contribution of CFP-LXXLL and ERα-RFP to Each Excitation/Emission Channel

Total background-subtracted fluorescence from each nucleus was quantified in each channel for each digital image. A, The amount of fluorescence measured in the red channel when only CFP-LXXLL was expressed (red from CFP) was minimal (0.09%, on average, of the amount of fluorescence in the cyan channel). 24.55% of the cyan fluorescent of CFP-LXXLL alone bled through into the FRET channel (FRET from CFP). B, Fluorescence bleedthrough of ERα-RFP-expressing cells in the cyan (cyan from RFP) and FRET (FRET from RFP) channels was 0.16% and 2.88%, respectively. These values were constant regardless of the amount of CFP-LXXLL or ERα-RFP transiently expressed in these cells. This demonstrates the accuracy by which these physical constants for each fluorophore was measured.

donor (FRET channel: 431–434 nm excitation/580–630 emission) relative to a decrease in donor fluorescence (cyan channel: 431–434/455–480). Therefore, FRET was measured as an increase in the ratio of FRET/cyan fluorescence from a cell expressing both CFP-LXXLL and ERα-RFP relative to the FRET/cyan ratios emitted from independently expressed CFP-LXXLL and ERα-RFP. In the control CFP-LXXLL-expressing cells, the amount of bleedthrough fluorescence into the FRET channel was 0.2455 ± 0.0094 that emitted in cyan channel (Fig. 2A), which did not vary with the overall amount of CFP-LXXLL in the cell (Fig. 3A, Xs). The bleedthrough of ERα-RFP fluorescence to the FRET channel was 0.0288 ± 0.0066 that emitted in the red channel (Fig. 2B), which also did not vary with the amount of ERα-RFP present in each cell (Fig. 3B, *black boxes*).

To calculate the relevant FRET/cyan from donor ratio, we first calculated the amounts of CFP-LXXLL and ERα-RFP present in the coexpressing cells. This was achieved by subtracting the minor contributions of CFP to the red channel (0.09% the value of cyan fluorescence using the matched control data in the prior section) and of RFP to the cyan channel (0.16% the value of the corrected red channel). We then subtracted the contribution of RFP to the FRET channel (2.88% the value of the corrected red channel). This remaining signal in the FRET channel contained the CFP bleedthrough to the FRET channel plus any sensitized emissions that resulted from the transfer of energy from CFP to RFP. If there was no FRET, the FRET/donor (remaining FRET/corrected cyan, hereafter FRET/cyan) ratio remained that of the donor CFP alone (0.2455). However, if there was transfer of energy from CFP to RFP, the amount of CFP fluorescence decreased and the amount of FRET increased, so that the FRET/cyan ratio increased.

The FRET/cyan ratio averaged from 32 E2-treated cells coexpressing CFP-LXXLL and ERα-RFP increased to 0.5412 ± 0.2018. Because cells with low amounts of CFP-LXXLL relative to ERα-RFP have fewer CFP donors in close proximity to the RFP acceptor, the FRET/cyan ratio varied with the relative amounts of RFP and CFP fluorescence measured in each cell. To account for this variation, the FRET/cyan ratio was graphed against the relative amounts of bleedthrough-corrected cyan and red fluorescence for each E2-treated cell (Fig. 4, *black boxes*). The slope of this graph was linear and consistent between experiments, indicating that the acceptor (RFP)-driven level of FRET within each cell was a constant. If the LXXLL peptide were not attached to CFP (Fig. 4, *gray triangles*) or if CFP-LXXLL and ERα-RFP coexpressing cells were treated with the antiestrogen ICI 182,780 instead of E2 (Fig. 4, *white boxes*), the FRET/cyan ratio remained identical to the 0.2455 FRET/cyan ratio of CFP-LXXLL alone (Fig. 4, Xs at acceptor/donor = 0) regardless of the relative amounts of CFP-LXXLL and ERα-RFP measured in the cell. This validated our calculations and demonstrated the accuracy with which we measure the energy transfer. Thus, we observed a ligand-regulated direct interaction of an LXXLL peptide with ERα in living cells.

### Ligand-Regulated, AF-2-Dependent FRET between GFP-LXXLL and ERα-RFP

To further validate our FRET measurements, we repeated the FRET studies of ERα-RFP with the same F6 LXXLL peptide, but labeled with GFP instead of CFP. Control measurements similar to those described above for the CFP-LXXLL construct were conducted to determine the bleedthrough of GFP-LXXLL fluorescence into the red and FRET channels. These GFP-LXXLL bleedthrough constants, and bleedthrough constants

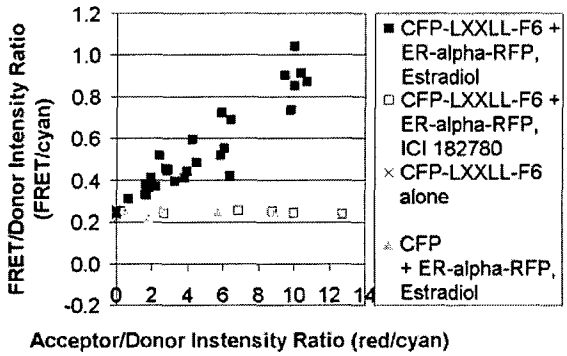
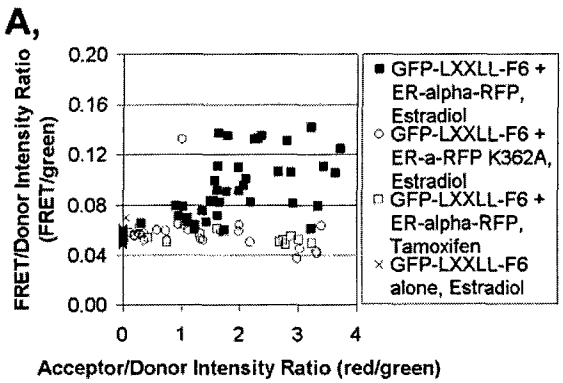



**Fig. 4.** Estrogen-Specific Interaction of CFP-LXXLL and ERα-RFP in Living Cells

Background-subtracted fluorescence from each nucleus was corrected for the bleedthrough values of red from CFP, cyan from RFP, and FRET from RFP (see *Results*). The remaining amount of fluorescence in the FRET channel isolates the contribution from the donor CFP to the FRET channel. It also contains emissions resulting from any energy transferred from CFP to RFP. Productive FRET also is accompanied by a decreased emission in the donor, cyan, channel. Thus, the FRET/cyan ratios from the isolated donor were calculated for each nucleus using the bleedthrough-subtracted values to determine the amount of energy transferred. These FRET/cyan ratios were plotted against the amount of acceptor (red) relative to donor (cyan). When the cells are treated with E2 (*black boxes*), the FRET/cyan ratio increased linearly with the red/cyan ratio at a slope characteristic of the efficiency of energy transfer from CFP to RFP. In contrast, the FRET/cyan ratio was not different from CFP-LXXLL alone (Xs) if the cells were treated with the SERM ICI 182,780 (*white boxes*) or if the LXXLL peptide was removed from CFP (*gray triangles*).



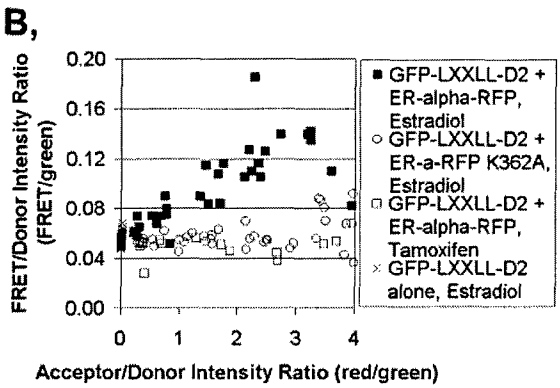




**Fig. 5.** AF-2-Dependent Interaction of Two Different LXXLL Peptides with ERα in Living Cells

FRET/Donor ratios were calculated for ERα-RFP interactions with two different LXXLL peptides (21, 31) attached to GFP: A, F6 (same as in Fig. 4) and B, D2. The FRET/donor ratio increased with the acceptor/donor ratio in cells treated with E2 (*black boxes*), but not tamoxifen (*white boxes*). Mutation of lysine 362 in the hydrophobic AF-2 cleft of ERα resulted in a loss of FRET (*white circles*) indicating that AF-2 was required for the direct interaction of LXXLL with ERα-RFP measured by FRET in living cells. Similar measurements were made for both peptides for different SERMs (Table 1).

determined from parallel ERα-RFP control cells, were used to calculate the amount of ligand-regulated FRET in cells coexpressing GFP–LXXLL and ERα-RFP. Coexpressing cells treated with tamoxifen showed a FRET/donor (FRET/green) ratio of 0.0530 ± 0.0043 (n = 18) (Fig. 5A, *white boxes*), that was not significantly different from the 0.0541 ± 0.0039 ratio measured in the control cells expressing GFP-LXXLL alone (Fig. 5A, Xs).

After treatment with E2, cells containing both ERα-RFP and GFP-LXXLL showed a FRET to green ratio of 0.0971 ± 0.0291 (n = 44) that varied proportionally to the RFP/GFP ratio (Fig. 5A, *black boxes*). This confirmed that E2 promotes an interaction between ERα and LXXLL in living cells, whereas tamoxifen does not. When E2-treated cells coexpressing ERα-RFP and GFP not containing the 19-amino acid LXXLL peptide were analyzed, the FRET/GFP ratio remained at 0.0549 ± 0.0098 (n = 14). In addition, mutation of a single lysine in the hydrophobic cleft of ERα to alanine (K362A) abolished FRET of GFP-LXXLL with ERα-RFP in E2-treated cells (Fig. 5A, *white circles*) as the FRET/GFP ratio remained as 0.0557 ± 0.0063 (n = 27). This demonstrated that the cleft, which is essential for E2-dependent transcription via AF-2 (25), is required for direct interaction of LXXLL with ERα in the physiological environment of the living cell.

In the presence of E2, the FRET/green ratio from coexpressed wild-type ERα and LXXLL increased with the RFP/GFP ratio in the cells (Fig. 5A), confirming that *bona fide* FRET was detected. The slopes of these graphs, summarized in Table 1 as the range of slopes encompassing the 95% confidence intervals, emphasized that interaction of the F6 LXXLL peptide with ERα was promoted by E2 (slope = 0.017 to 0.021), but not by the SERMs tamoxifen (slope = −0.001 to 0.001) or ICI 182,780 (slope = −0.002 to 0.000). Thus, FRET precisely measured a ligand-specific, AF-2-dependent direct interaction between ERα and an LXXLL peptide in living cells.

### Ligand-Specific Interactions of Different LXXLL Peptides Binding to AF-2 in ERα

FRET measurements are highly sensitive to distance between the fluorophores, and fall off to the sixth

**Table 1.** Ninety-Five Percent Confidence Intervals in Slopes and Y-Intercepts of FRET/Donor *vs.* Donor/Acceptor Graphs (RFP/GFP <4)

| | GFP-LXXLL-F6 | | GFP-LXXLL-D2 | |
|---|---|---|---|---|
| | Slope | Y-intercept | Slope | Y-intercept |
| No ERα-RFP | No slope | 0.053–0.055 | No slope | 0.053–0.055 |
| ERα-RFP wt | | | | |
| No hormone | 0.007–0.011 | 0.053–0.057 | 0.007–0.009 | 0.053–0.056 |
| *E2* | *0.017–0.021* | 0.053–0.058 | *0.022–0.026* | 0.053–0.058 |
| Tamoxifen | −0.001–0.001 | 0.054–0.055 | −0.005–0.003 | 0.053–0.055 |
| ICI 182,780 | −0.002–0.000 | 0.054–0.055 | −0.001–0.002 | 0.054–0.055 |
| *DES* | *0.016–0.021* | 0.052–0.057 | *0.018–0.023* | 0.053–0.057 |
| *EIA* | *0.017–0.021* | 0.053–0.056 | *0.014–0.018* | 0.053–0.057 |
| *DHF* | *0.017–0.021* | 0.053–0.056 | **0.029–0.034** | 0.052–0.055 |
| ERα-RFP K362A | | | | |
| No hormone | −0.004–0.001 | 0.054–0.057 | −0.005–0.003 | 0.054–0.055 |
| *E2* | −0.003–0.001 | 0.054–0.059 | 0.001–0.003 | 0.053–0.056 |
| *DES* | −0.004–0.002 | 0.054–0.056 | 0.002–0.006 | 0.053–0.056 |
| *EIA* | −0.001–0.002 | 0.053–0.056 | 0.000–0.003 | 0.054–0.056 |
| *DHF* | −0.007–0.002 | 0.054–0.056 | 0.000–0.003 | 0.054–0.055 |

power as the distance between them increases (28, 29). The distance dependency of FRET would, in principal, allow the detection of small conformational differences between interacting molecules. We therefore measured the level of FRET between ERα-RFP and another 19-amino acid-long, LXXLL-containing peptide, "D2" (21), fused to GFP. D2 contains sequences flanking the LXXLL motif that differ from those in the F6 peptide.

Control measurements established that the FRET/GFP and RFP/GFP ratios for GFP-LXXLL-D2 alone were no different from those measured for GFP-LXXLL-F6 (not shown). When coexpressed with ERα-RFP, GFP-LXXLL-D2, like GFP-LXXLL-F6, showed E2- and AF-2-dependent FRET that was not promoted by tamoxifen (Fig. 5B). In the presence of saturating ($10^{-6}$ M) E2, the slopes of the FRET/GFP *vs.* RFP/GFP graphs (reported as 95% confidence intervals) were similar, but slightly different, for GFP-LXXLL-F6 (Table 1, 0.017–0.021) and GFP-LXXLL-D2 (0.022–0.026). This suggested that the D2 and F6 peptides bound to the hydrophobic cleft of E2-bound ERα with marginal differences in orientation or with slightly different affinities.

### Using FRET to Distinguish SERM-Regulated Interactions

The ability of FRET to measure subtle differences in the direct interactions of the LXXLL motif and ERα in living cells could be used as a sensitive new assay for detecting specific activities of new SERMs *in vivo*. All ligands that trigger LXXLL motif binding to ERα are known to strongly activate transcription at promoters containing the classical ERE promoter element. Therefore, a compound that elicits a strong level of FRET between ERα-RFP and GFP-LXXLL in this assay might also activate transcription of an ERE-driven gene in a reporter assay.

To test this hypothesis, we synthesized and tested two synthetic ligands of uncharacterized estrogenic or antiestrogenic activities that were reported previously to bind ER with high affinity (38–40): ethyl indenestrol A and 6,4′-dihydroxyflavone (Fig. 6). These compounds and a known ER agonist diethylstilbestrol were compared with E2 for their abilities to elicit FRET in cells coexpressing ERα-RFP and GFP-LXXLL-F6 or GFP-LXXLL-D2. E2, DES, EIA, and DHF all were able to trigger significant levels of FRET between ERα-RFP and both LXXLL peptides (Table 1, slopes, *italic*). All of these ligand-regulated interactions were blocked upon mutation of lysine 362 in ERα to alanine (Table 1, ERα-RFP K362A), indicating that LXXLL was interacting directly with the hydrophobic pocket of ERα in each case.

For interaction of ERα-RFP with GFP-LXXLL-F6, the levels of FRET activated by E2, DES, EIA, and DHF were not statistically different ($P > 0.05$) (Table 1, GFP-LXXLL-F6 slopes). Similarly, the levels of FRET determined for GFP-LXXLL-D2 interaction with ERα-RFP in the presence of DES and EIA were not significantly different than those observed with GFP-LXXLL-F6 (Table 1). In contrast, DHF activated a significantly greater level of FRET with the D2 LXXLL peptide than with the F6 LXXLL peptide (Table 1, *boldface*). Thus, all compounds promoted the direct interactions of two different LXXLL peptides with ERα in the cellular environment, but precise FRET measurements allowed subtle variations in those interactions to be observed.

The similar levels of FRET with the F6 peptide suggested that the ER-binding compounds EIA and DHF both caused ERα to adopt a conformation that permitted LXXLL-F6 to bind into the hydrophobic AF-2 cleft in the same orientation as occurs when E2 or DES binds to ERα. Because reporter gene assays show that DES and E2 activate transcription from a classical ERE in an AF-2-dependent fashion, the similar AF-2/LXXLL-F6 interactions adopted by the EIA and DHF-bound ERs suggested that these compounds might activate transcription at an ERE site. Reporter gene

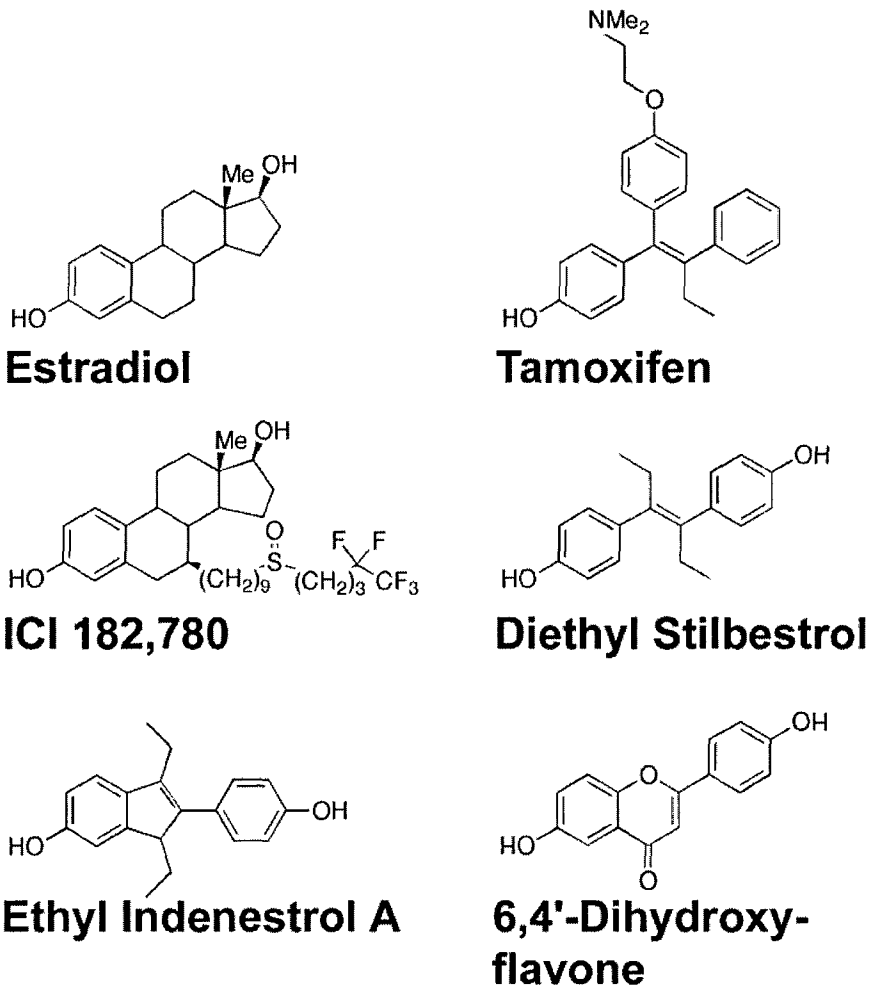



**Fig. 6.** Structures of the ER-Binding Compounds Used in this Study

The phenolic ring common to all compounds is oriented to the *left*. There are two different phenolic rings in ethyl indenestrol A and 6,4′-dihydroxyflavone, for which only one of the orientations is shown.

assays were performed in HeLa cells using transiently transfected wild-type ERα and a luciferase gene driven by the classical ERE from the vitellogenin promoter (Fig. 7). The promoter was activated upon expression of unliganded ERα. This activation was blocked by the SERM raloxifene, which acts as an antiestrogen for AF-2-dependent transcription (41). Incubation with $10^{-5}$ M EIA and $10^{-5}$ M DHF both activated transcription from an ERE site as effectively as $10^{-5}$ M E2. Thus, the ability of two different LXXLL peptides to productively interact with ERα AF-2 in living cells was associated with agonist activity of four separate ligands, each with a distinct chemical structure.

## DISCUSSION

The ligand-regulated interactions of a receptor with its cofactors are fundamental to nuclear receptor action (3, 15, 42–44). These interactions are commonly detected with *in vitro* column-binding assays that rely on the interactions of purified proteins in artificial buffers. Alternatively, two-hybrid assays detect an ill-defined cellular association between two proteins as the activation of a downstream reporter gene. As more is understood about the mechanisms underlying nuclear receptor activation, new challenges are arising to effectively and efficiently measure those interactions, particularly in living cells (31, 45–49).

Only recently has FRET been used to measure protein-protein interactions in the environment of living eukaryotic cells (28, 49, 50). FRET has been used to detect nuclear receptor interactions with cofactor fragments, labeled with spectral variants of GFP (49). We show here that LXXLL peptides by themselves are sufficient to interact, in an agonist-regulated fashion, directly with ERα in the cellular environment (Table 1). Moreover, these interactions are wholly dependent upon AF-2 in ERα. A weak interaction of LXXLL with ERα was also detected in the absence of ligand. This

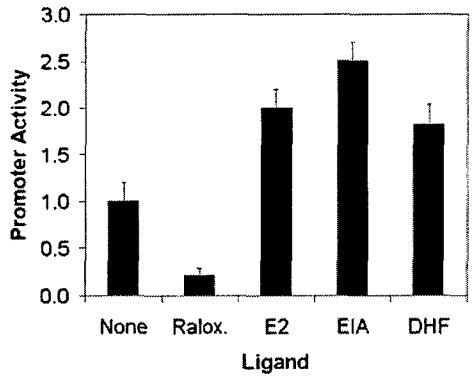



**Fig. 7.** ER Binding Compounds that Promote FRET also Activate a Simple ERE-TATA Promoter

HeLa cells transfected with an expression vector for wild-type ERα and with an E2-regulated promoter were treated with different ligands. Raloxifene blocked promoter activation by expressed ER interacting with estrogens in the cell culture media. E2, EIA, and DHF all caused further activation of the promoter.

ligand-independent interaction was blocked by the same K362A mutation in AF-2 that abrogated agonist-dependent FRET. Thus, in living cells, both the ligand-independent and agonist-dependent interactions of LXXLL with ERα are dependent upon AF-2.

Our approach allowed us to precisely quantify the level of FRET between the interacting factors. Because the amount of FRET falls very rapidly, to the sixth power, with the separation of the fluorophores (28–30), differences in the relative spatial orientations of ER and LXXLL affect the amount of FRET measured in each complex. In our studies, the efficiency of FRET between ERα and a given LXXLL peptide was similar for each ligand. However, there were subtle differences, particularly with 6,4′-dihydroxyflavone for which the level of FRET was higher for ERα interaction with LXXLL-D2 than with LXXLL-F6. The slight variations in the levels of FRET indicate subtly different ligand-specific interactions. Different levels of FRET may suggest that the LXXLL peptide is bound to AF-2 in a different position such that the GFP and RFP fluorophores are different distances apart. Alternatively, the on- or off-rates for the interaction of LXXLL with ERα may be different, leading to quantitatively different levels of FRET. For instance, the higher level of LXXLL FRET with all liganded ERs than with the unliganded ER is consistent with prior observations that agonist binding dramatically stabilizes the LXXLL interaction with ERα (31, 51).

The FRET studies described here, by themselves, showed the similarities and differences in LXXLL interactions with ERα bound by four different activating ligands and two different AF-2-blocking ligands. Other techniques may complement the FRET studies of ligand-selective nuclear receptor action. Determining the kinetics of fluorescence recovery after photobleaching fluorophore-linked nuclear receptors (46, 48) and cofactors (48) at isolated regions within the nucleus may help to determine if the ligand-selective changes in FRET efficiency arise from altered kinetics in the LXXLL interaction with ERα. Measuring the recovery of FRET after selectively photobleaching the interacting cofactor would more precisely correlate fluorescence recovery with direct interactions with ERα in the cellular environment.

The benefits of drugs that regulate nuclear receptor activities in some tissues are often counteracted by unwanted receptor actions in other tissues. It is therefore important to identify compounds with desirable selective modulatory properties (12, 13, 20–22, 41, 52). However, most current assays for interaction are insufficient to distinguish the tissue-selective actions of new compounds from previously existing SERMs (22, 41). The precise measurement of ER/cofactor interactions afforded by FRET will allow the detection, in different cellular environments, of more subtle differences in the interactions of ER, or any other nuclear receptor, bound to different ligands. This will aid the development of clinically effective compounds that regulate specific interactions in specific cell types. Indeed, we found that FRET between ER and two LXXLL-containing peptides was useful in predicting the E2-mimicking activity of two previously untested ER-binding compounds, EIA and DHF. However, different levels of FRET for the LXXLL interactions with AF-2 suggest that these compounds possess somewhat distinct properties. This precise quantification of FRET between nuclear receptors and conformation-specific peptide probes developed by us (20–22) and others (53) will greatly contribute to a better mechanistic understanding of estrogen action and may be potentially useful for discovering SERMs with improved tissue-selective actions.

## MATERIALS AND METHODS

### Analysis of Estrogen-Regulated Promoter Activation

The 3xERE-TATA-Luc and C3-Luc estrogen-responsive promoters have been previously described (32, 33). The carboxy terminus of human ERα was fused, in frame with an eight-amino acid linker, to the amino terminus of RFP by inserting a PCR-generated ERα cDNA into the *Nhe*I and *Bam*HI sites of pDsRed1-N1, an RFP expression vector (CLONTECH Laboratories, Inc., Palo Alto, CA). The ERα-RFP expression vector was cotransfected with either the 3xERE-TATA-Luc or C3-Luc reporters into HepG2 cells and the transfected cells were treated with E2, tamoxifen, ICI 182,780 or ethanol control vehicle as previously described (21). Cells were then lysed and the amount of luciferase activity in the extracts was measured as previously described (21).

The synthesis, ER binding properties and transcriptional activation profiles of EIA and DHF will be reported elsewhere. Transfection conditions and assay protocols used for the testing of EIA and DHF with wild-type human ERα and the ERE reporter gene assay in HeLa cells (Fig. 7) were identical to those reported previously (41, 54).

### Cellular Imaging

GHFT1–5 cells were transfected with the ERα-RFP expression vector and doxycycline-inducible GFP-LXXLL or CFP-LXXLL expression vectors as previously described (31). Transfected cells were grown for 24 h in estrogen-free media containing 3–5 μg/ml doxycycline. A total of $10^{-6}$ M of each ligand was then added and the cells grown for a further 24 h before data collection. Quantitative fluorescence images were collected with a Hamamatsu ORCA cooled interline camera attached to an Olympus Corp. IX-70 microscope controlled by Universal Imaging Corp. (Downingtown, PA) Metamorph software. Filter combinations, described in the Results section, were obtained from Chroma Technology Corp. (Brattelboro, VT). Fluorescence quantification of marked nuclei and background was performed using Metamorph software. Background subtractions and bleedthrough corrections were applied using Microsoft Excel (Microsoft Corp., Redmond, WA).

### FRET Analysis

FRET measurements can be accomplished using relatively affordable fluorescence microscopic equipment and image collection software. Control measurements of the cells separately expressing the donor or the acceptor fluorophore (Fig. 2) indicate the point at which the user's equipment no longer accurately quantifies the fluorescence ratios critical for FRET determination. Only cells containing donor and acceptor fluorescence amounts greater than those that are accurately measured are included for calculating FRET. Because the ratios measured are physical parameters of the fluorophores, FRET measurements are highly consistent between separate experiments provided that all parameters affecting the relative ratios of fluorescence quantification in the donor, acceptor and FRET channels are kept constant. This includes using the same 1) objective lens, 2) relative integration times for the different channels, 3) dichroic mirror, 4) excitation/emission filters, and 5) camera.

Average fluorescence ratios ± SD were calculated from data collected on multiple days using Microsoft Excel. Ninety-five percent confidence intervals in the slopes and Y-intercepts of the FRET/donor *vs.* acceptor/donor graphs were calculated using GraphPad Software, Inc. Prism software (San Diego, CA). Only data up to an acceptor/donor ratio of 4 were included in the calculation of the slopes for GFP-LXXLL FRET with ERα-RFP. The linearity of the graphs tended to decrease beyond this point, as the amount of acceptor became more saturating. This acceptor/donor ratio of 4 should not be used by others as a defined parameter as it depends on the nature of the molecular interaction (our unpublished data) and the ability of the user's equipment used to quantify the specific acceptor and donor fluorescence.

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